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CYTOCHEMICAL STUDY RELATED TO LASER APPLICATION

Final Report

December 1978

by

K. C. Tsou, Ph. D.

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University of Pennsylvania School of Medicine
Philadelphia, Pennsylvania 19174

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Using electronsytochemical methods for NADH diaphorase, Mg++-ATPase, Na+-K+ ATPase, the effect of CO_2 laser on Rhesus monkey cornea and its safety threshold level has been investigated.

It was found that both the endothelial cells and the epithelial cells are viable at 3-6 watts/cm^2 and 100 msec level, but there is functional alteration with respect to ion transport and the process of hydration. Whether such alteration could affect the integrity of the cornea, especially in chronic

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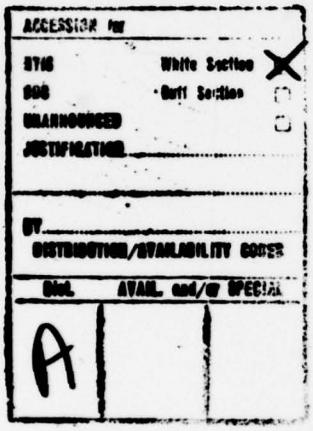
It was found also that at 20-30 watts/cm^2 and 100 msec level the repair of the epithelium is very efficient. This repair process involves the extrusion of the dead epithelial cells in the center and the movement of regeneration cells at the peripheral region towards the center. The most important ultrastructural observation is made that the wing cells on the upper layer of the epithelium function in a clock-like synchronization to remove dead cells and thereby insure the clarity of the cornea. This process is energy dependent and does require ATPase. This process is probably not limited to laser damage,

The observations and conclusions derived may be tentative because of limitation of available samples, but should be amenable to further experimentation.



Abstract

Using electroncytochemical methods for NADH diaphorase, Mg++-ATPase, Na^+-K^+ ATPase, the effect of CO_2 laser on Rhesus monkey cornea and its safety threshold level has been investigated.

It was found that both the endothelial cells and the epithelial cells are viable at 3-6 watts/cm^2 and 100 msec level, but there is functional alteration with respect to ion transport and the process of hydration. Whether such alteration could affect the integrity of the cornea, especially in chronic effect should be of concern to the laser safety program.

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The observations and conclusions derived may be tentative because of limitation of available samples, but should be amenable to further experimentation.

INTRODUCTION

The use of laser has been on the increase in recent years. Protection against hazards created by the use of this new versatile tool in modern science has hence become necessary. The current consensus standard is that of ANSI Z136.1-1973 [1]. The Army has also set its own standard [2,3], and the Bureau of Radiological Health [4] has also studied the criteria related to laser safety. While guidelines and control procedures have been stipulated by these agencies, fundamental biological effects data are required for evaluating exposure criteria for laser radiations [5,6,7].

In the area of research related to the safety of the eye, cornea deserves investigation, since retention of a smooth, transparent cornea is of major importance in maintaining normal vision [8]. The integrity of this surface could very well be challenged particularly for infrared lasers ($\lambda \geq$ 1.4 microns) where the laser energy is absorbed by the cornea or outer ocular media. Clinically, such damage could be checked through visual inspection with a slit lamp. If a permanent visual opacity is produced at the time of the radiation insult or develops as a chronic effect, a corneal transplant may be required [9].

Since the current safety standard for infrared lasers is based upon slit-lamp observation of corneal changes, it seems logical to ask whether one could detect corneal alteration at doses below that required to produce such acute effects and at the current doses considered safe. Functional alteration of the cornea at the cellular level requires investigation. The effect of CO_2 laser radiation at 10.6 microns is of special concern because of its use in industry, medicine, and the military. When one considers the limitation of available experimental material (Rhesus monkey

cornea), there is no current biochemical technique suitable for such a study. Enzyme cytochemical methods therefore represent the only promising approach to such investigation. This report therefore summarizes our recent work in this area.

MATERIALS AND METHODS

1. Rhesus monkey corneas were exposed to various doses of CO_2 laser, ranging from 3 watts/cm^2 to 65 watts/cm^2 for a duration of 100 msec. The irradiance diameter was 3.2-3.8 mm. range. Rabbit corneas were used also in the methodology development phase to ascertain optimal conditions. There were noticeable differences between Rhesus monkey cornea and rabbit corneas.

2. Preparation of Tissues - Initially, the corneas were freshly excised and stored in liq. N_2 until ready for cytochemical study. This procedure proved to be unsatisfactory because of the difficulty in avoiding ice artefacts in unfixed tissues. Unfixed tissues were considered useful for the study of succinic dehydrogenase and cytochrome c; both these enzymes are unstable if the tissues were fixed. When realizing the limitation of the material and the importance of preserving as good morphology as we could, we decided to use glutaraldehyde fixed material, and use NADH as marker enzyme for mitochondria activity. A 3% glutaraldehyde in 0.25 M sucrose was found suitable as fixative for NADH diaphorase and Mg^{++}-ATPase study. Phosphate buffer could be used for NADH diaphorase, but not for Mg^{++}-ATPase. For Na^{+}-K^{+} ATPase, a 2.5% glutaraldehyde in 0.05 M cacodylate buffer could be used. Details will be seen in each individual procedure, together with their incubation conditions.

The corneas were also cut into three areas to facilitate study under the electron microscope (Fig. 1). The lesion was designated area 1. The halo(visible) region surrounding the lesion, formed by the shrinkage of tissue as a result of the heating effect, was designated as area 2. The periphery of the cornea became area 3. Further division of the quadrants to a, b, c, d, e, f could also be used to designate the abnormal area found during light microscope study, if any. This system would theoretically permit the reconstruction of enzymatic profile of the cornea and make possible morphometric study, even though time did not allow us to carry out such detailed studies in depth.

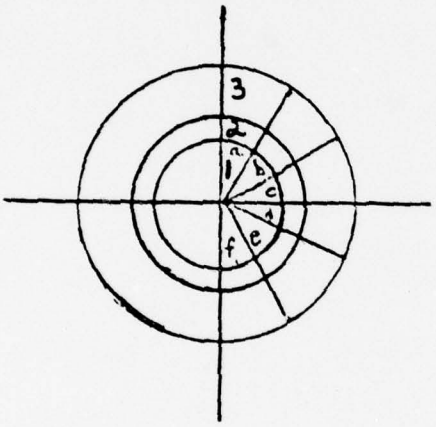
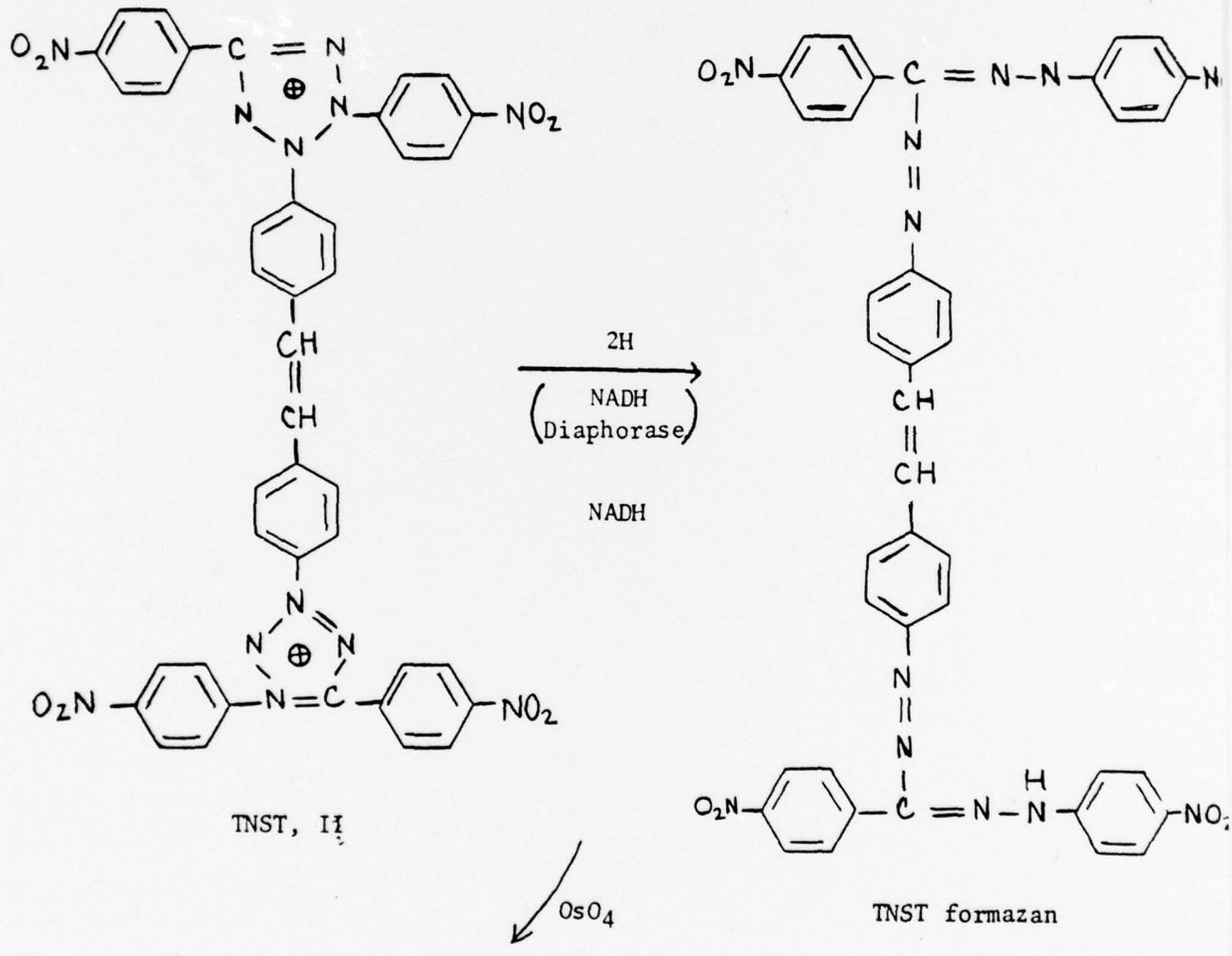


Fig. 1 Rhesus Monkey Cornea - Demonstrates mini lesion
ca. 28 w/cm^2, 100 msec, 1 hr. after lasing

3. Cytochemical Procedures

a) NADH (Reduced nicotinamide adeninediphosphate) Diaphorase Study.

Even though light microscopic methods for the demonstration of this enzyme was developed (by us) sometime ago (10), a substrate with better penetration and more sensitive for this enzyme in glutaraldehyde fixed-tissue was needed. After testing various substrates synthesized in our laboratories (11), we decided to use TNST (2, 2', 5, 5'-tetra-p-nitrophenyl-3, 3'-4, 4'-stilbene ditetrazolium chloride, I). This reagent was successfully tested in glutaraldehyde fixed tissue and the formazan formed could be osmicated with osmium tetroxide (1% O_sO_4 in phosphate buffer or 0.25 M sucrose). The formazan formed is red, and when reacted with osmium tetroxide, it turned purplish and is stable throughout the processing steps for electron microscopy (see chart for formula).

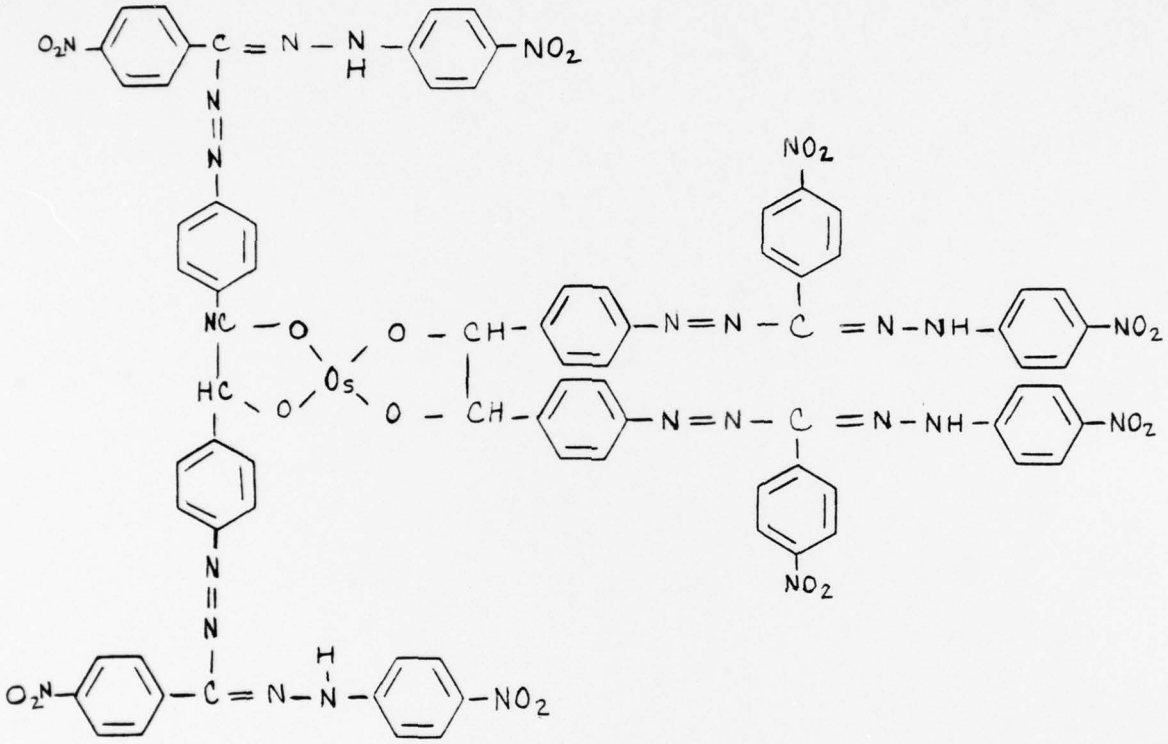


TNST, II

TNST formazan

(red)

(continued from preceding page)



Insoluble Os - formazan (purplish green)

Procedure: The laser treated cornea and half of the control cornea were fixed for one hour in 3% glutaraldehyde in phosphate buffer (0.05 M, pH 7.4) or in 3% glutaraldehyde in 0.25 M sucrose at room temperature. After rinsing in sucrose to remove excess glutaraldehyde, they were incubated in the following medium:

TNST	10 mg	
NADH (Wako)	20 mg	-- The purity of NADH is very important in these experiments. The best grade is that made by Wako.* Chemically reduced NADH which contains sulfite should not be used.
Phosphate buffer (0.1 M, pH 7.4)	10 ml	
Sucrose	1.25 g	

Red color could be seen in the control as well as the treated cornea. Incubation time initially was set at 30' because of the higher activity observed in the laser treated samples. It was subsequently lengthened to 1 hr. for 20-40 watts/cm^2 and 1 1/2 hrs. for 3-10 watts/cm^2 samples. After rinsing in buffer or sucrose to remove unreacted substrates, the corneas were treated with 1% osmium tetroxide in phosphate buffer for two hr. at room temperature, then dehydrated through alcohol grades as usual and embedded in an Epon mixture (30 A:70 B). Flat embedding molds were used and polymerization of the embedded tissue was carried out at 65^{o} for three days. Ultrathin sections were obtained with an MT-2 Ultramicrotome and examined with an RCA EMU-4A electronmicroscope. Thick sections of 1 μ were cut to sandwich the thin sections to ascertain the region of interest.

*Wako Chemicals, P.O. Box 29346, Dallas, Texas 25229.

b) Na^+-K^+ ATPase

Preparation of 5-Nitroindoxyl Phosphate

A mixture of 0.437 g of 5'-nitro-N-acetyl indoxyl in 15 ml dry pyridine was treated with 0.2 ml $POCl_3$ (12). The mixture was allowed to stand at room temperature overnight. The reaction was stopped by adding 50 ml of cold 7 N ammonium hydroxide. The mixture was again allowed to stand overnight at room temperature. The solvents were removed by evaporation under reduced pressure. The residue was mixed with 200 ml water and the pH of the solution was adjusted to 8.0. The insoluble material in the solution was removed by filtration and the filtrate was purified with a Sephadex A-25 anion exchange column. A product of 1.5 g was recovered from the column and isolated as ammonium salt. The product was hydrolyzed completely to nitroindigo by *E. Coli* alkaline phosphatase at pH 9.0.

Procedure

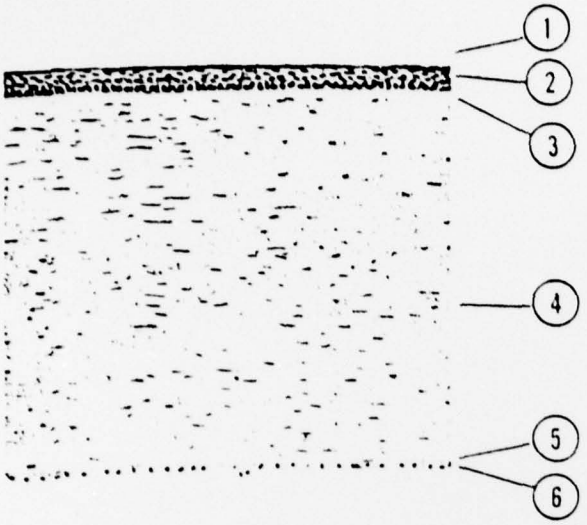
Cornea was fixed in 2.5 - 3% glutaraldehyde in 0.05 M cacodylate buffer, pH 7.4 for one hour and rinsed in sucrose (0.25 M) and then incubated in the following medium:

5-Nitroindoxyl phosphate	5 mM
$SrCl_2$	5 mM
Tris buffer (0.1 M, pH 9.0)	4 ml
$MgCl_2$	10 mM
KCl	10 mM
Levamisole (10 mM)	0.25 ml

$SrCl_2$ alone, ouabain (5 mM), and without Levamisole are used as control. The difference between the use of Levamisole and without the use of Levamisole (an alkaline phosphatase inhibitor) was not much in Rhesus monkey cornea.

RESULTS

Normal cornea morphology is shown in the following scheme (13).



Light micrograph of section of central cornea. Numbers indicate layers:

1) tear film; 2) epithelium;

3) Bowman's membrane; 4) stroma

5) Descemet's membrane; 6) endothelium.

Under the light microscope, the cornea is divided into three layers: the epithelium, the stroma and the endothelium. Under the electron microscope, there is a difference between the wing cells and the basal cells in the epithelium. The Bowman's layer shows hemidesmomembrane structure. The endothelium is situated adjacent to the Descemet membrane and facing the aqueous humor. Sometimes, a light layer of carbohydrates can be seen facing the aqueous side of the endothelium (Fig. 2a, 2b, 2c & 3a).

NADH Diaphorase

The NADH disphorase study has been reported previously (14). The result of this study is therefore combined with the Na^+-K^+ ATPase in the accompanying Table I and essentially summarized our observations from these limited samples.

TABLE I

SUMMARY OF NADH DIAPHORASE AND Na^+-K^+ ATPase in RHESUS MONKEY CORNEA ENDOTHELIUM AFTER LASER*

Exposure Levels (w/cm^2)	3	6	10	25	32	65
NADH Diaphorase	weak activation	weak activation	weak activation	strong activation	damage & activation	extensive damage but strong activation
Na^+- K^+ ATPase	activation	activation	n.d.	n.d.	n.d.	n.d.

*1 hour after lasing; 24 hours repair complete at level greater than 3 watts/cm^2. Vacuolization greater in all lasing samples, but lacked dose response, controls are much weaker than treated.

Na^+-K^+ ATPase (15,16)

While several methods were examined for this enzyme (17,18,19), we decided to choose the 5'nitroindoxyl phosphate method because only in this method was there sufficient difference between the epithelium and the endothelium.

At 3-6 watt/cm^2 level, this method has provided the most interesting observation for Na^+-K^+ ATPase. As shown in Fig. 6 the endothelium shows activation in lasing area for one cell and inactivation in the adjacent cell. No higher or lower energy level has been studied, but the control sections show relatively weak activity and no alternate activity difference was observed.

In the endothelial cells, the localization of the enzyme on the plasma membrane was also considered. In the majority of pictures, we observed darker staining on the *inner* side of the plasma membrane, but occasionally intercellular deposits have been seen which may be related to the long incubation times in an active region.

Epithelial activity of this enzyme is not completely negative; while most cells are devoid of the plasma membrane activity whether in the center of lasing or not, plasma membrane activity appears occasionally in basal cells. Plasma membrane activities are definitely strong in the wing cells.

DISCUSSION

As mentioned in the earlier reports, the limitation of the number of samples in this type of experiment is obvious. In addition, judicious choice of dose response experiments is nexessary since it is also not possible to do too many experiments in the dose range required. Yet, in spite of all these difficulties, the use of electron microscopy and electroncytochemical methods has paved ways to better our understanding of the functional alteration of the cornea after lasing with CO_2 laser.

1) Viability

The NADH diaphorase experiments have shown that the viability of the endothelium sustains at 10 watts/cm^2. There may be questions at 3-6 watts/cm^2 level, but in some high substrate concentration experiments, we could see activity in these cells. Whether NADH diaphorase activity could be used alone as a viability index may be subject to question, but we have initially explored NADH, NADPH, and succinic dehydrogenase systems in cornea. Only NADH activities are sufficiently high to be observable under lasing.

The other important question yet to be settled is the obvious increase in activity after lasing at high dose level 20-65 watts/cm2. These could be seen even at light microscope level. At electron microscope level, they appear also related to the electrondense deposits around the tonafilaments. Most of these tonafilaments form "clumping" after lasing. This is a definite phenomenon not obvious from the light microscope study.

These non-mitochondrial reducing activities of the TNST may reflect some change at the chemical level of the tonafilaments related to this clumping. One attractive hypothesis is the scission of disulfide bond after lasing, which then reduces the tetrazolium salt to an electrondense product. At this time,

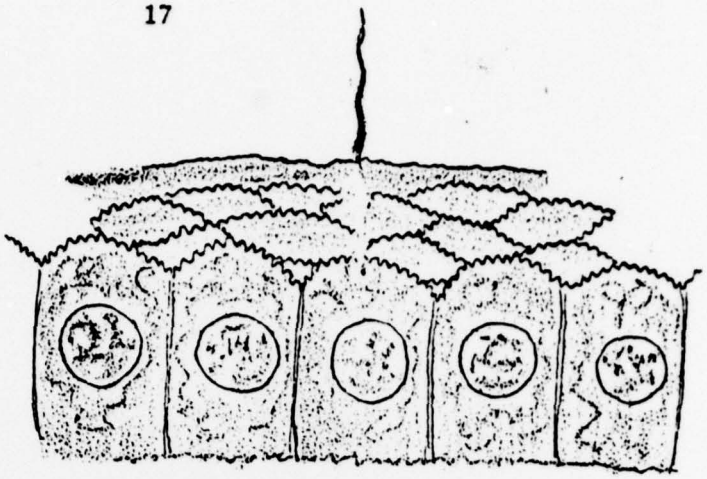
there is not enough chemistry study in this area to support our assumption. We could study this problem in the future.

After 24 hours, the damage to these epithelial cells was repaired, but tonafilament "clumping" did not disappear if the monkey eye was not taped (covered). If the monkey eye was taped, we noticed such clumping was absent in basal cells. Thus, it might be suggested that this clumping of tonafilaments may not be due to heat alone, but is part of the natural process of the cornea to screen out UV or other unnecessary radiation. This suggestion also deserves further study, as this is the first evidence of the function of these filaments in the basal epithelial cells (non-collagenous type). The ability of basal cells to synthesize collagenous fiber is another subject of interest. We did not notice any difference, however, in the present study.

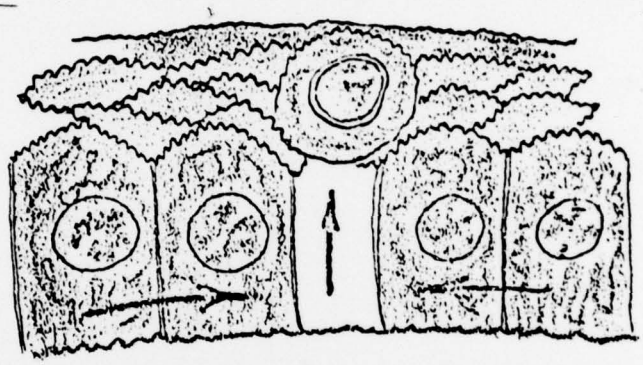
2) Repair Process (18)

One of the difficult problems in the repair mechanism is the role of the wing cells in the epithelium. In this study, we clearly demonstrated the extrusion ability of these cells in removing dead cells and that the process is largely played by the wing cells (see diagram on page 17). The question then naturally arises as to what is the source of energy to make this process possible. Is it mitochondrial ATPase activity or Ca^{++} activated ATPase in wing cells? Our Mg^{++}-ATPase study tentatively supports the presence of such ATPase activity in the mitochondria of the wing cells, and that they increase in number in the peripheral region. An obvious experimental difficulty lies also in the increase in vesicular bodies that are responsible for trapping dust and other undesired particles. The evidence today is that these are more the role of the cells near the sclera region, and occasionally phagocytic cytolysosomes may be found.

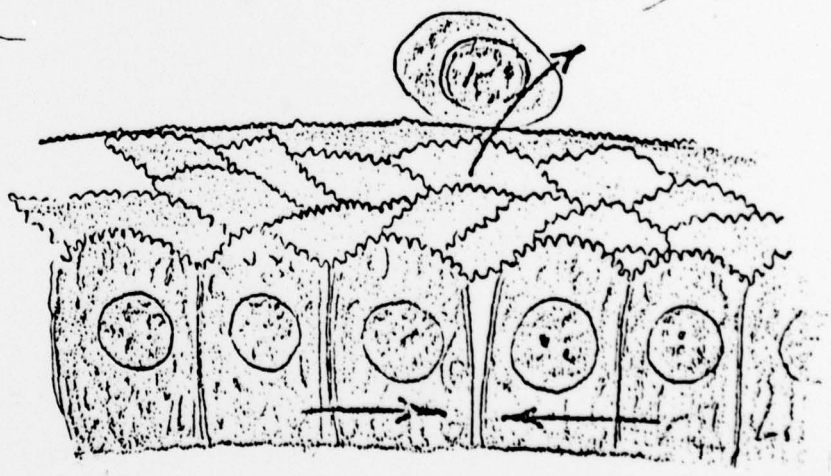
A. Damage in Center



B. Cell Extrusion



C. Removal of Necrotic Cell



3) Hydration and Regulation of Water (20)

The primary effect of lasing is likely to produce heat, even though the alteration of protein structure should also be considered. Since we do not know about the chemical alteration by CO_2 laser radiation at present, our discussion of the effect will have to concentrate on corneal hydration. While it is commonly known that Na^+-K^+ ATPase controls the ion flow, it is of importance to realize that this process also regulated the hydration process because of the fact that both Na^+ and K^+ carry water molecules in this pumping process. This is especially important in sudden alteration of the cellular environment by a drastic process such as lasing.

While the endothelial cells remain attached to the Descemet membrane at these threshold conditions, it is important for us to observe and realize that there is functional alteration associated with the vacuolization phenomenon. We assume that vacuolization in these experiments is related to the CO_2 laser radiation, as normal Rhesus monkey cornea had been observed in the past to have relatively low (but not absent) vacuolization, if excised properly.

Based on the above observations, it could be postulated tentatively that the effect of CO_2 lasing on Rhesus monkey endothelium involves the following steps: 1) local transmission of heat through the epithelium layer, the stroma layer, the Descemet membrane to the endothelial cells. The fact that it manifests more readily in endothelial cells may be related to the higher water content in these cells rather than specific higher absorption of this unicellular layer; 2) This process initially activates the ATPase "pump" near the aqueous humor side and may inactivate the ATPase pump near the Descemet membrane side. The result of this process is probably to bring water into the cells, which may be related to the vacuolization

process. There is often ATPase activity around these vacuoles in our experiments.

3) The adjacent cell is obviously also affected by the lasing, but may turn off its pump completely in order to allow maximum efficiency of the neighboring cells. This may be analogous to the fact that in fire fighting, one tries to save one house from the neighboring house by abandoning the effort in one house; 4) An alternate possibility would be an oscillatory mechanism whereby these two adjacent cells could work alternately to activate and inactivate the ATPase pump and so to speak, "cool" each other in the process. This is a very attractive postulate and has support in other oscillatory mechanisms in biochemistry; 5) There is a possibility that fusion of two cells could take place as a repair mechanism; 6) Alternately, the fusion of part of the intercellular plasma membrane may provide water and ion flow from one cell to the other as a repair mechanism. Our observation that ATPase is active in the juxtaposition may lend support to such a mechanism.

REFERENCES

1. American National Standard Institute report, N.Y., N.Y.

2. U.S. Department of the Army, 1974. Control of Hazards to Health from Laser Radiation, TB MED 279, U.S. Government Printing Office, Washington, D.C.

3. D.H. Sline, Evaluating Health Hazards from Military Lasers, J. Am. Med. Assoc., 214 (6) 1047 - 1054, 1970.

4. Bureau Radiological Health Bulletin #PP 32259, Table I-A. 1975.

5. E.S. Beatrice and G.D. Frish, Retinal laser damage thresholds as a function of image diameter. Arch. Environ. Health 27: 322-326, 1973.

6. N.F. Gamaleya in Chapter on Laser Biomedical Research in the USSR., p. 133, in "Laser Applications in Medicine and Biology" vol. 3, ed. by M.L. Wolbarscht, Plenum Press, N.Y., 1977.

7. K. Rowe and R.J. Rockwell, Investigation of Ocular Hazard from Lasers in Human Subjects, Final Report USAF Contract F 41609-69-C-0052, 1972.

8. P. Payrau, Y. Pouliquen, J.P. Faure, and G. Offret: La transparence de la cornee. Les mechanismes de ses alterations, Paris, 1967, Masson & Cie.

9. H. Katsin, and J.F. Meltzer: Combined Surgery for corneal transplantation and cataract extraction, Am. J. Ophthal. 62: 556, 1966.

10. M. Yanoff and K.C. Tsou, Demonstration of ocular reduced Diphosphopyridine nucleotide (DPNH) and triphosphopyridine nucleotide (TPND) diaphorases using tetrazolium salts. Am. J. Ophthal. 310-317, 1965.

11. Synthetic procedure for this new tetrazolium salt was originally developed by Dr. B. Seamond and Dr. D. Lynm; prepared by Dr. K.F. Yip and Dr. K.W. Lo for these experiments.

12. This modified procedure by Dr. K.F. Yip is based on the original method from D.J. Rabiger, M.Y. Chang, S. Matsukawa and K.C. Tsou, J. Heterocyclic Chem. 7: 307, 1970.

13. B.S. Fine and M. Yanoff, Ocular Histology, P. 142, 1972, Harper and Row, New York. --Dr. Fine's discussion of the ultrastructure of cornea has been beneficial to our work.

14. K.C. Tsou, P. Shwaluk, B. Stuck and E. Beatrice, Proc. EMSA, 1974.

15. K.C. Tsou and M. Khatami, A new cytochemical method for Na^+-K^+ ATPase. Proc. Histochem. Soc. 1976.

16. K.C. Tsou and M. Khatami, Ultrastructural demonstration of transport ATPase in Rabbit cornea, Proc. EMSA, 1977.

17. K.C. Tsou, K.W. Lo, and K.F. Yip, A new method for adenosine triphosphatase adaptable for fluorescent and electronmicroscope application. J. Histochem. and Cytochem. 23: 303 - 306, 1975.

18. K.C. Tsou, B. Stuck, and E. Beatrice, Proc. EMSA, 1976.

19. M. Wachstein and E. Meisel, On the H istochemical demonstration of glucose-6-phosphatase. J. Histochem. Cytochem. 4:592, 1956.

20. K.C. Tsou, B. Stuck, S. Shuschereba, and E. Beatrice, Proc. J. Marshall Symposium on Cell Biology, Dec., 1977 (Philadelphia).

LEGEND

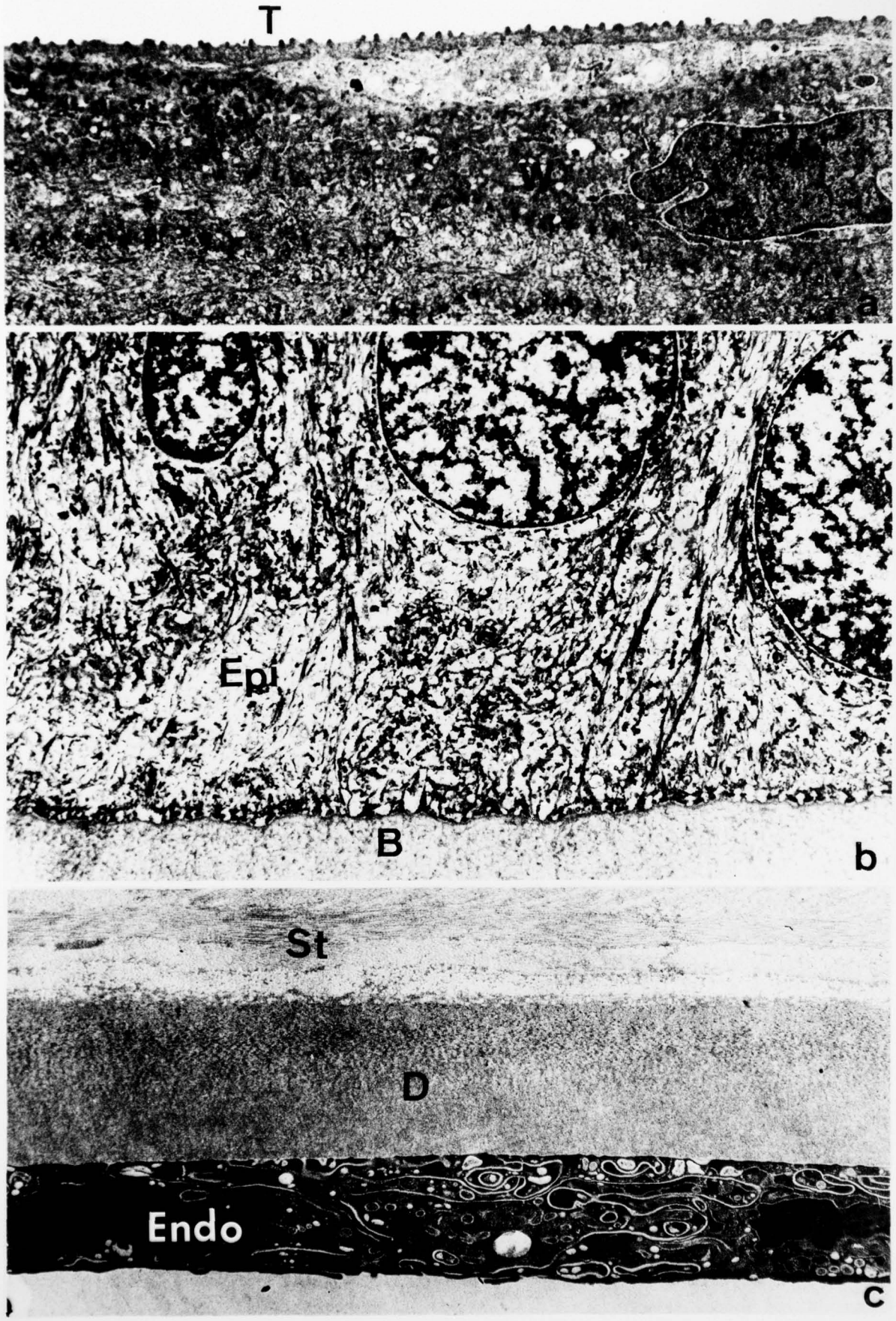
Fig. 2a Rhesus monkey cornea, wing cell layer in epithelium. (T)-tear layer, (W)-wing cell, uranyl acetate stain, 11, 100 x

Fig. 2b Rhesus monkey cornea, basal epithelial cells (Epi) and Bowman's layer (B). uranyl acetate stain, 11, 100 x

Fig. 2c Rhesus monkey cornea, posterior stroma region, (St),Descemet membrane (D) and endothelium (Endo) uranyl acetate stain, 11, 100 x

Fig. 3a Rhesus monkey cornea, Bowman's layer (B) and anterior stromal region. uranyl acetate stain, 8300 x

Fig. 3b Rhesus monkey cornea, threshold lasing, Na-K-ATPase activity, note heterogenous stain of two adjacent cells in vicinity of center of lasing. (D) Descemet membrane, (A) Aqueous humor, 22,000 x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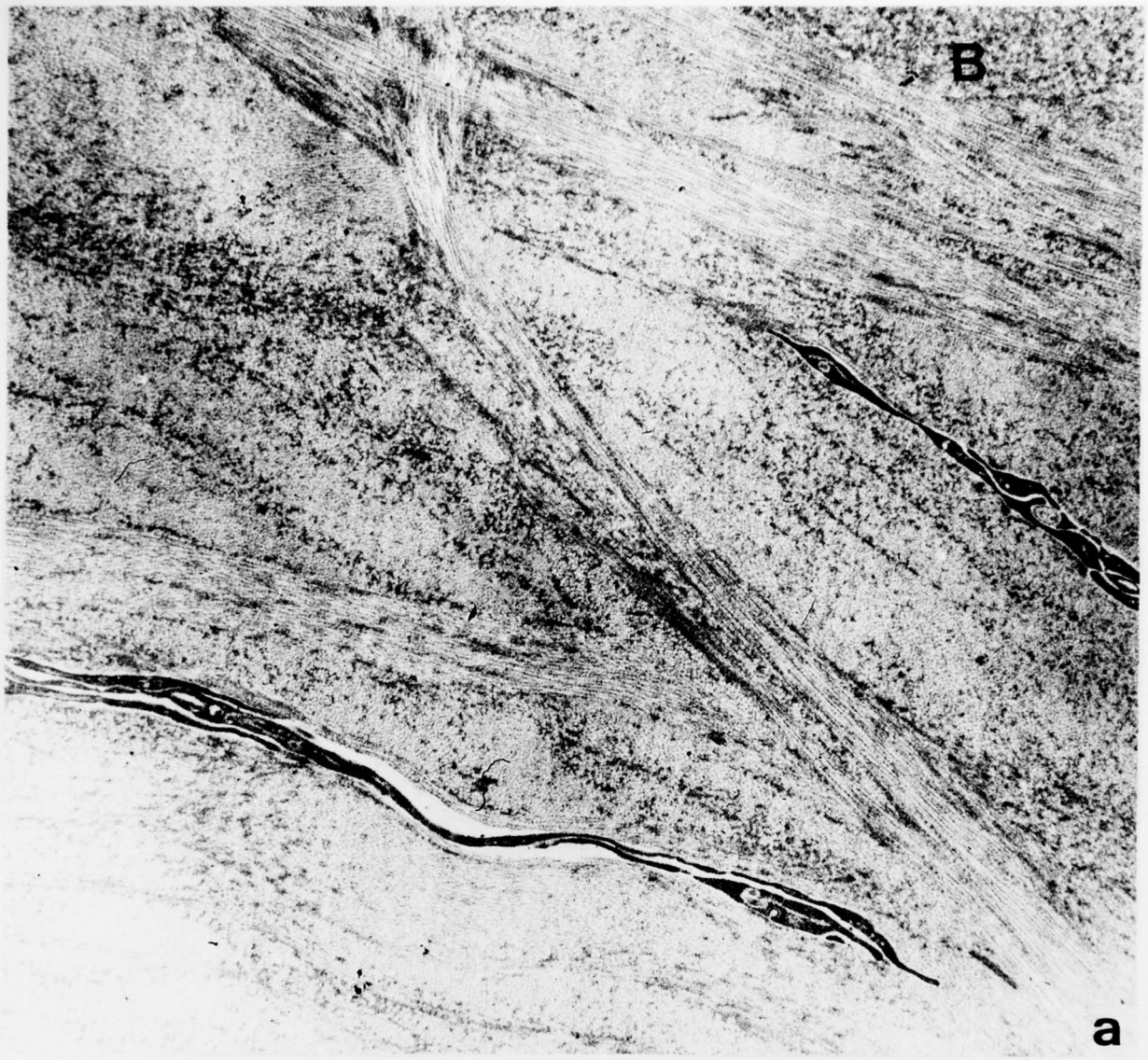
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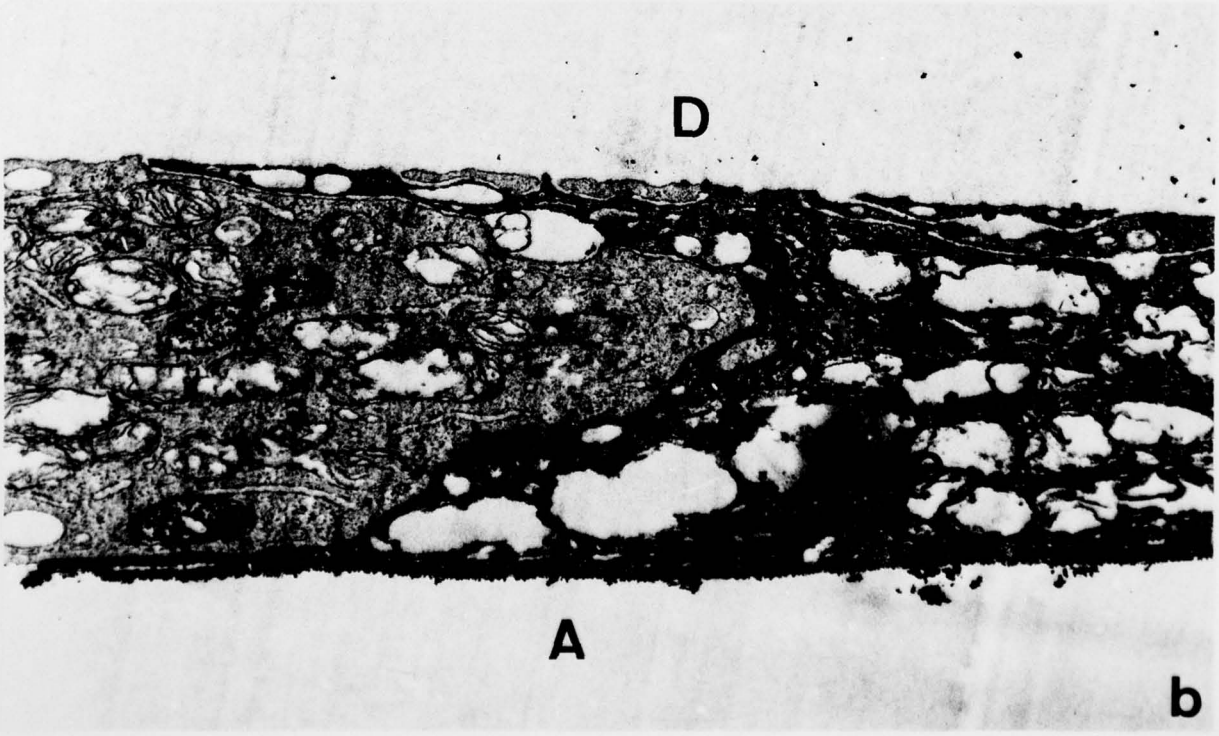
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